The Marine Laboratory

UNIVERSITY OF MIAMI

54-5

Final Report
January, 1954

Marine Borer Investigations

Office of Naval Research

Contrace Nonr 705(OC)

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CORAL GABLES, FLORIDA

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University of Miami

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Coral Gables
Florida

F. G. Walton Smith
Director

ML-6635

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SUMMARY

This final report presents the results of the investigations conducted by this laboratory during the past five years. The results fall naturally into three main categories:

(1) Natural History
(2) Physiology and Biochemistry
(3) Creosote Studies

Anatomy, Histology, Life History and Metamorphosis. Our studies have confirmed the oft-repeated statement that Teredo is but a highly specialized lamellibranch mollusk. It began its existence as a fertilized ovum which is apparently retained in the maternal mantle cavity. It implants itself into the substance of the maternal gill and there completes the development essential to prepare the larva for a free-living existence. Once freed from the maternal gill the larval Teredo enjoys a very brief free-swimming period of existence. During this 48 to 72 hour time it has not been observed to feed. After having settled, fortuitously, upon a suitable wooden substratum, the larva actively burrows into the wood, undergoes metamorphosis and reaches sexual maturity within 21 to 28 days. The average duration of its life, based on studies of 250 animals, is but ten weeks.

Effects of Environmental Agents. During its adult life Teredo is effectively insulated from its environment except for the necessary respiratory circulation which it maintains through the siphons. Hence environmental components have their most pronounced effects on the larval forms. In local waters temperature does not appear seriously to influence either settling of larvae or growth of the adults. In a similar way the concentration of oxygen at all depths and in all seasons is not limiting either to larval distribution or to growth of the adult. In local waters so long as the salinity exceeds approximately 10o/oo, such as might be found in or near fresh water outfalls, there is no effect on Teredo. The larvae appear to be quite insensitive to osmotic effects. It has been observed, for example, that larval Teredo will continue actively to crawl in a saturated sea water solution of magnesium sulfate. They have been observed to crawl over still undissolved crystals with undiminished activity. Under the conditions obtaining in these studies, current velocities between 1.4 and 1.8 knots prevented entrance of Teredo larvae into wooden panels. The reaction of the organisms to conditions of illumination varies with the age of the larvae. 12-hour larvae are apparently insensitive to light. At later developmental stages they are stimulated to swim actively in the presence of light. The larvae have been shown to resist gravitational effects by active swimming. This would tend to cause them to accumulate in the surface layers of water. The photic response described above, combined with the gravitational response, would lead to greater infection of surface wood during the hours of darkness.

Physiology and Biochemistry

Biochemical Analysis of Adult and Larval Forms. Protein nitrogen determinations of an extensive series of adult forms of all sizes have

indicated that the total N content of Teredo is low -- of the order of 1.5 to 2.5 percent. Analysis of the wood in which Teredo lives have demonstrated that this source is probably not adequate to account quantitatively for the nitrogen observed in a fully grown Teredo. Amino acid analyses have further shown the wood to be deficient in certain amino acids which are regularly found in the borers. Examination of nannoplankton has revealed the missing amino acids probably to have been contributed from this source. Hence, it is thought, the final total N found in Teredo, originates both from wood and from nannoplankton introduced in the respiratory stream of water.

Teredo, in common with certain other lamellibranchs, is characterized by a high glycogen content. In the adult this frequently may constitute 50% of the dry weight of the animal. Teredo has been shown to possess the enzymatic equipment necessary to convert the wood and other cellulosic material of its immediate environment into glucose and then into glycogen. It has been shown that the glycogen content of the unfertilized ovum is high; that glycogen is the chief fuel used in the preattachment activities of the free-swimming larva and that, subsequent to invasion of wood, the glycogen content of the post-larval form increases. If Teredo is removed from wood and kept alive in sea water, the glycogen content of the animal is decreased at least 75% in 10 days. This observation would indicate that the maintenance of normal glycogen levels requires the continuous ingestion of cellulose. In the adult borer glycogen is stored in the gonads, the mantle, the siphonal musculature and in the adductor muscles of the shells.

Analyses have been made of total phosphorus, total calcium and of Iron, in addition to the glycogen, and nitrogen studies previously mentioned. Total phosphorus of the pallets is at least twenty times as large as the total phosphorus of the shells. There appears to be no regional localization, within the body of the worm, of organically bound phosphorus. The shells consist almost entirely of calcium. The total iron content of the shipworm is concentrated in the visceral mass. As might be expected because of the concentration of enzymes and other protein materials in the visceral mass, the total nitrogen content of this portion of the borer is higher than that of the remainder of the animal.

Physiology of adult and larval forms.

Oxygen consumption of adult borers, both in situ and after having been removed from their wooden matrix, has been studied in an apparatus designed in this laboratory for this specific function. Oxygen consumption varies inversely with the total size of the animal, ranging from 0.099 to 0.250 ml O_2/hr/gm dry weight. Borers which have been removed from wood show a significant depression of oxygen consumption. A capillary microrespirometer has been designed to accommodate a single larva of Teredo, and to permit observation of oxygen consumption for extended periods of time. It has been shown that oxygen consumption of the free-swimming larva is highest at 24 hours post-spawning and thereafter decreases abruptly until 72 hours. After this time there is a more gradual diminution in the rate of oxygen consumption until three hundred hours, at which time the larvae, denied access to wood, are all dead. It is

thought that the progressive decrease in rate of oxygen consumption is related to the continuous utilization of glycogen in swimming and crawling.

Pumping rate and hydrodynamic mechanism: The magnitude of the cilia-driven respiratory current in Teredo has been directly measured and indirectly estimated by two different methods. Values determined by these procedures agree within 8 percent. The average value for ventilation rate was found to be 4.1 liters of sea water per hour per gram of dry weight. This is nearly four times the average rate reported by various investigators for the oyster. Direct cannulation of the mantle cavity of living Teredo in situ has shown that the ciliary and sphincteric mechanisms are capable of maintaining a positive intra-mantle pressure of about 5 mm. water. The importance of this positive pressure is difficult to overestimate. Thus, it is because of this factor that the adult animal is enabled to keep the boring shells in close apposition to the advancing end of the tunnel. The positive intramantle pressure also makes it possible for the animal to prevent fortuitous contamination of the mantle cavity.

Cellulase Enzyme system: We have shown that Teredo, both adult and larva, possess a powerful cellulase enzyme complex. This enzyme system is responsible for conversion of the wood, which is rasped away by the shells in the boring process and passed through the gut of the borer, into glucose which can be used for metabolic fuel. The presence of the cellulase enzyme system in the larva is of importance in the initial penetration of the wood. In its conditions of activity, that is pH, temperature salts etc., this enzyme has been shown to be similar to that of the cellulase of the mold Myrothecium verrucaria.

Boring mechanism: The internal turgor required to keep the boring surface of the shells in apposition with the advancing face of the tunnel has been shown to be the result of the activity of the cilia of the mantle and the gill in maintaining a positive intramantle pressure. The actual boring is accomplished by shell teeth, which develop shortly after the initial penetration of the wood by the larva. The shells are powered by well-developed adductor muscles. These muscles contain very high concentrations of glycogen. Their functional activity appears to be continuous throughout the life of the borer.

Carbohydrate metabolism: Preliminary analyses have indicated that Teredo contains many of the standard intermediates of the phosphorolysis of glycogen. Thus, hexosephosphates, adenosine and adenosine polyphosphates, arginine phosphate and adenylic acid have all been identified both qualitatively and quantitatively in homogenates of shipworms. It is particularly interesting to observe that the phosphagen which is present in Teredo is arginine phosphate instead of the creatine phosphate which is so typical of the phosphate reserves of vertebrate animals.

No final conclusion can be reached as yet regarding the food of Teredo: there is evidence to support the contention that wood provides all of the energy required and most of the nitrogen. There is also evidence that some, at least, [illegible]e amino acids which exist in the adult shipworm must have had their genesis in dietary nannoplankton, since they have not been

detected in the wood in which the borers live, and since they probably can not be synthesized by the animals. It appears most likely that *Teredo* uses both nannoplankton and wood for food.

X-ray studies of the growth *in situ* of over 250 individual borers supports the following generalizations: the average length of life is but 10 weeks; growth is continuous during this period; the attainment of sexual maturity is signalized by a characteristic distortion of the growth curve; there appears to be little if any seasonal variation in the total rate of growth.

CREOSOTE STUDIES

Biological Assay Procedures. Since the rate of oxygen consumption provides a sensitive index of physiological condition it was determined to study the effects of creosote upon the respiration of individual larvae of *Teredo*. Larval teredids are sensitive to extreme dilutions of whole creosote in sea water. Concentrations of 5×10^{-8} gms. creosote/ ml of sea water produced a decrease in oxygen consumption of 68.8 percent. Since creosote is a complex of ill-defined chemical entities, some of which are probably completely innocuous, other components of the mixture must be extremely toxic. Efforts were therefore directed toward a clarification of the pharmacology of creosote and of its component fractions. Capillary microrespirometry proved to be too precise and too tedious for routine large-scale testing. Accordingly a new method was developed, employing the equally wide-spread crustacean borer *Limnoria lignorum* as a test form, and studying the effects of creosote upon its rate of respiration as revealed in the standard Warburg respirometer. Some creosote fractions supplied to us by the Naval Research Laboratory were shown to be no more effective in increasing oxygen consumption of Limnoria than is m/1,000 glucose. On the other hand two solvent extraction fractions produced the same respiratory response as whole creosote at comparable dilution. The most effective fraction so far studied was "Creosote with Tar Bases Removed". This produced an increase of over 100% in oxygen uptake over the rate of untreated normal animals. These results demonstrate that this method has definite utility in differentiation between creosote preparations, and will vastly accelerate the procedure of pharmacological assay.

Accelerated leaching tests: It was early observed that creosote-treated wood liberated something to the sea water which was detrimental to shipworm larvae. The assumption appeared to be justified that the protection conferred upon wood by creosote-impregnation was related to the liberation of toxic materials to the sea water. Conditions were sought under which the natural rate of release of toxic components of creosote from impregnated wood might be reproducibly accelerated. The first baths studied included boiling sea water, alkaline potassium dichromate, hydrochloric acid, sodium hydroxide and cold aerated sea water. Most reproducible results were secured with mild leaching agents. Exposure of test slips for 16 days in an 80°C fresh-water bath was finally established as standard. Test slips exposed to this standard leaching procedure were then evaluated by field exposure in the ocean for a standard period of six months and were then rated in accordance to borer damage they had suffered. There is a

general agreement between the results of microrespirometer studies of toxicity of creosote fractions and the results obtained in this study of accelerated leaching. Information on the physical behavior of creosote and of its component fractions in wood will be of great utility in planning rational and practical preventive measures.

NATURAL HISTORY OF TEREDO

Life History and Metamorphosis

Prior to the studies completed in this laboratory there were very few observations on the larval stages of shipworms except for early veliger stages and stages subsequent to attachment. No information was available for any stage of Teredo pedicellata. Investigations in this laboratory have included free-swimming stages up to the period of metamorphosis of this species. A detailed study of the gross morphology and behavior from the later swimming period up to the early boring period has been completed.

Laboratory culture methods have been developed which have provided large numbers of larvae of known age for study. The second generation of captive Teredo has been obtained on several occasions. Breeding has been found to occur during all months of the year but to show considerable variation in intensity. The maxima are in early summer and late fall. Larvae are released, for the most part, at night.

The bivalve shell of the newly spawned larva is globular with an average diameter of 245 ($\pm$ 20) microns, and entirely contains the contracted soft parts of the larva. The frontal diameter is equal to, or slightly less than the antero-posterior diameter of the shell. The larval shell is cuticular in composition and light purplish-brown in color. No evidence of calcareous components was observed until several hours after the young Teredo had begun excavations in wood.

Immediately upon release from the excurrent siphon of the parent the larvae begin active swimming. The velum is the sole swimming organ with

the shell not being used in any way as a locomotor organ. Swimming is mostly in a vertical direction. Actively swimming larvae make progress at a rate of about 7.7 mm per second upwards and about 7.0 mm per second downwards. The specific gravity of the living larvae is greater than that of water.

By 48 hours the larvae begin to crawl over suitable substrates, or even on the surface film of the water. Crawling takes place as a result of a considerable extension of the tip of the foot. The tip becomes attached and the body is drawn forward by a muscular contraction of the entire foot. The rate of crawling movement is in the order of 5 mm per minute.

Attachment takes place between 36 and 72 hours and is generally mediated by the foot. This organ is provided with a pair of large ventral glands which stain deeply with intra-vital neutral red. No byssus thread is produced by this foot gland, although they are probably homologous with structures described by Sigerfoos in 1908 as the "byssal gland". Attachment is usually to spring wood and only occurs if the wood has been conditioned in seawater.

After attachment the distal end of the foot sweeps in all directions around the young shipworm. It is clearly seen to initiate boring by scraping off the loose surface fibers and cells of the wood. This detritus accummulates as a mucus ring which gradually develops into a cone covering the burrow, except dorsally where the siphons protrude. If this cone be removed mechanically, it is reformed within eight hours.

Shortly after the mucus ring is formed, the valves begin to take part in boring. They are rocked from side to side by a movement of the entire animal rather than by any action of the adductor muscles, so that first

one valve and then the other is brought into contract with the wood. Orientation also changes from time to time through 90° in the horizontal plane. Calcareous deposits accumulate in the cone, which is completely calcified by the fifth day, when the functional pallets first appear. Although teeth develop on the shell at the time of attachment, they do not become calcified until calcification of the mucus cone is initiated. The change in axis of the shell movement from the larval to the adult type does not occur until several days after attachment, when the foot undergoes a marked shortening and thickening. The foot is then virtually a sucking disc. By this time the mantle edges have fused.

Metamorphosis follows a similar course even if the larvae are denied access to wood. As a result of this, the animal is unable to enter wood unless it reaches a suitable surface in less than five days after release from the mantle, due to changes in the foot which will then prevent initial penetration.

Further growth takes place as the tunnel proceeds and the valves sink below the surface. This growth results in an increase in length of the animal. The initial boring is at right angles to the surface of the wood and only later changes direction. The burrow, once its definitive direction is established, thereafter tends to run parallel with the fibers of the wood.

Effects of Environmental Agents

Water Currents. To assess the influence of current velocity upon the settling of Teredo, larvae a four-sided wooden tube of square cross-section and eight feet long was constructed. The sides diminished in width gradually from one end to the other. The dimensions varied, in different experiments from 1.5 inches square at the small end to 8.0 inches square at

the large end. This tube was attached to an electric pump by its small end to produce an apparent velocity range of from about 0.1 to 2.2 knots. At the conclusion of an experiment the box was dismantled and the component sides were studied as individual panels.

The results show clearly that borer attacks are greatly reduced by an increased rate of flow in the surrounding water. A current velocity between 1.4 and 1.8 knots prevented entrance of Teredo. This is readily explained as a purely mechanical effect of the water movement. When the velocity of the ambient water exceeds either the speed of swimming of the larva or its ability to adhere to the substrate it will be denied the opportunity to penetrate the wood. These results suggest that except in crevices or in places where stream pockets occur, borer attack is not likely to take place in wooden vessels under way, or during periods of strong tidal flow while at anchor. On the other hand, once the borer has entered it is protected by its burrow from any subsequent flow of water. It must be concluded therefore, that, because of the intermittent nature of tidal flow, the mooring of a vessel in a tideway offers little or no protection against borer attack.

Temperature. Many previous workers have stated that low temperatures are detrimental to the activity of borers and that prolonged exposure will eventually result in their death. Investigation of the temperature factor in local waters was made by means of maximum-minimum thermometers which were placed in the water, at both the surface and the bottom, at each of the twelve stations which were studied. The lowest minimum temperature was 17.5°C in June at one station. The average temperature of all stations lies close to 25°C. It is doubtful whether the temperature factor, which is an important factor in northern water, is critical here. The warmest month

coincides with the greatest attack rate. Apart from this quantitative effect, temperature appears not to be a limiting factor for our waters.

Salinity. Since the range of salinity found in the existing stations is comparatively small both seasonally and by stations it is doubtful whether this factor has any major influence on the growth or attack cycle of the borers in this area. Monthly salinity readings were obtained by means of a hydrometer at each of the stations.

Table I

Monthly Salinity Readings ($^{o}/oo$) Obtained at Selected Panel Stations (Readings obtained with Hydrometer)

	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Station 2										
Surface	36.5	37.3	38.0	39.1	34.0	34.0	36.4	33.8	34.2	34.6
Bottom	36.5	36.9	38.2	39.0	34.6	34.8	37.2	35.2	34.8	34.6
Station 12										
Surface	24.8	26.6	34.0	28.4	20.8	13.0	22.0	20.1	19.5	10.3
Bottom	35.3	34.3	37.2	31.8	22.9	33.2	25.2	28.4	26.5	26.6
Station 5										
Surface	36.8	34.3	38.0	39.6	38.7	37.6	37.0	32.0	34.1	33.8
Bottom	36.6	34.2	38.3	38.8	38.8	36.8	36.7	32.1	33.9	33.7
Station 9										
Surface	35.8	35.8	36.8	38.1	36.8	36.2	36.0	--	--	--
Bottom	35.8	35.7	37.0	38.0	29.3	36.2	36.1	--	--	--

Table I shows the range of salinities observed during one seasonal precipitation cycle. Shown also is the variation between surface and bottom.

Illumination. Response of Teredo larvae to various conditions of illumination is complicated by concurrent and often opposite, reactions to other environmental factors. Thus, it is that the basic motor response to light may be obscured by a simultaneous negative geotaxis which would tend to result in the congregation of the larvae in the upper few centimeters of

the water column. In an effort to elucidate the effect of illumination and to separate the effects of this factor from others acting at the same time, an extensive series of experiments has been performed on larval Teredo at various stages of development, beginning immediately after release from the mantle cavity of the parent.

In the first series of experiments on larvae it was found that 12-hour animals remained distributed at random in a dish, one half of which was in darkness and the other illuminated at a level of 67 foot candles. 48 hour larvae, on the other hand, redistributed themselves so that 71.5 $\pm$ 5%, were in the lighted portion of the dish.

In a second series of experiments the light source was so arranged that the larvae were obliged to swim towards the light in order to reach darkness. In both cases, at all ages and over a wide range of light intensity, up to 70% of the larvae moved away from the light source, even when this brought them into darkness. This indicates a negative phototaxis is present in conditions where directional illumination exists. The experiment described previously, however, shows that a negative photokinesis also exists, whereby the larvae tend to collect under conditions of light and to migrate from dark areas, during their later stages of development.

The behavior under natural conditions is complicated by a response to gravity. When kept in the dark the larvae tend to collect at the surface of a vertical column of water, and to maintain their position there by means of the activity of the velum. Upon exposure to light, either from above or below, most of the larvae swim downwards. When the light is turned off, an upwards movement again takes place. Younger larvae react to light in fewer numbers than do the older larvae. Greater activity in darkness is also supported by the observation that larvae studied in the dark demonstrate a

higher rate of oxygen consumption than do similar animals studied under normal laboratory conditions of illumination.

In summary then, the larvae appear to swim actively in the absence of light, but to reduce their activity in the presence of light. This results in a tendency to collect in lighted areas. The predominant upward swimming movement also tends to bring larvae to the surface under conditions of darkness. A negative photokinesis tending to cause aggregation in the lighted areas and a negative phototaxis tending to cause migration away from lighted areas would explain the observed greater attack in a certain range of illumination. Negative phototaxis should prevent attachment at high illumination intensities, while at lower, subthreshold, intensities the larvae would tend to swim actively upwards rather than to settle.

PHYSIOLOGY AND BIOCHEMISTRY

Biochemical Analysis of Adults and Larvae

Nitrogen Content and Distribution. A preliminary elementary analysis of Teredo was undertaken to provide information on which an energy balance sheet could be based, as well as to broaden the basis of our understanding of the biology of the animal. Total phosphorus, total calcium, iron and total nitrogen analyses have been performed. These studies have been made on entire animals and upon various parts of animals in order that some components might be localized with greater geographical precision. The following generalizations seem to be justified. (1) the inorganic components of the pallets appear to be predominantly phosphorus compounds, (2) total phosphorus is virtually identical in the visceral mass and in the balance of the worm. The level here is in the neighborhood of 1.0%. (3) the shells consist almost entirely of calcium. (4) the total iron content of the ship-

worm (ca 32 mg/cent) is concentrated in the visceral mass. The total nitrogen content of the visceral mass exceeds that of the remainder of the worm which is to be expected because of the greater concentration of enzymes and nitrogen-bearing tissues in this locus.

The amino acid composition of Teredo of all ages has been studied in relation to the amino acid composition of the wood in which it lives and to the composition of nannoplankton that might qualify as food organisms for Teredo. The results of these qualitative analyses are presented in Table II.

Table II

Qualitative Amino Acid Content of Teredo and of Organic Components of its Environment

Material Hydrolyzed	L	PA	V	T	P	M	A	AR
12-hour Larvae	X	O	X	O	X	X	X	X
Teredo less than 50 mg*	X	X	X	X	X	X	X	X
Teredo more than 50 mg	X	O	X	X	X	X	X	X
Pine Wood	X	O	O	O	O	X	X	X
Nannoplankton	X	O	X	X	X	O	O	O

* weights are dry-basis
L - leucine; PA - phenylalanine; V - valine; T - tyrosine;
P - proline; M - methionine; A - alanine; AR - arginine

It will be observed that the wood is deficient in phenylalanine, valine, tyrosine and proline, all of which appear in hydrolysates of adult shipworms. Hydrolysates of nannoplankton contain all the amino acids which are present in the shipworms but which are missing from the wood, with the sole exception of phenylalanine. Larvae differ from the adults qualitatively only in the

lack of the aromatic amino acids phenylalanine and tyrosine. These data suggest that the nitrogen present in the adult shipworm probably came both from the wood in which it lives and from nannoplankton swept into the mantle and so into the gut by way of the respiratory stream.

Glycogen Content and Distribution. A systematic study of the distribution of glycogen in the *Teredo* of all sizes has shown that this material may constitute up to 50% of the total dry weight of the animal. The average concentration shown in an extensive series was 30%. This figure is achieved within six weeks after the borer first invades wood. Having established the existence of very considerable concentrations of glycogen in *Teredo*, its location in the animal was next investigated. An initial survey was conducted in which different regions of the worms were separately analyzed for glycogen. One sample consisted of the gut and its contents, gut derivatives and the gonads from a series of worms. The eviscerated residues of the same animals formed the second sample. A third sample consisted of intact teredids. The glycogen content of the visceral sample averaged 0.12%, the eviscerated residue contained 19.3% and the intact control worms showed 23.57% glycogen. These results suggested that the chief glycogen depots in the animal were located elsewhere than the viscera. For more precise localization of the glycogen stores, a series of animals was prepared for histochemical study according to the method of Best. As might have been suspected from the generally high concentrations of glycogen which characterize *Teredo*, glycogen was found to be very widely and generally distributed through the sections of the animal. Noteworthy among the organs of the body for their extremely high concentrations of discrete particulate glycogen masses were the mantle, muscle tissues, gill and imbedded larvae.

The mantle contributes significantly to the total stores of glycogen contained in the animal. The muscles of the body, particularly those of the shells, the pallets and the siphons, all show considerable concentrations of glycogen. The siphonal musculature is particularly striking because it contains most of the glycogen of the siphon. In general the excurrent siphon contains more glycogen than the incurrent siphon. The musculature of the pallets is exceeded in its glycogen content only by the gill and imbedded larvae.

Physiology of Adult and Larvae

Oxygen Consumption. The conventional Warburg apparatus has several notorious shortcomings for the determination of the rate of respiration of the intact adult borer. Of these perhaps the greatest is the severe mechanical stimulation produced by the necessary agitation. It was found that *Teredo*, when subjected to the agitation produced by even 50 excursions per minute, retracted the siphons, extruded the pallets and essentially ceased actively to respire. Moreover, the small amount of fluid employed in the usual Warburg vessel was insufficient to provide an adequate respiratory flow.

Accordingly attempts were made to modify a principle first proposed by Scholander. In the apparatus finally devised, (Figure 1) the respiring organism is maintained in an adequate volume of aerated water, is not subjected to mechanical stimulation and can be observed continuously during the course of the hour-long respiration run. In essence the method makes use of a closed system in which the liquid and the gas phases are maintained in constant equilibrium by means of a rapidly revolving turbine, partly immersed in the aqueous phase, which constantly and thoroughly mixes the air and the liquid phases.

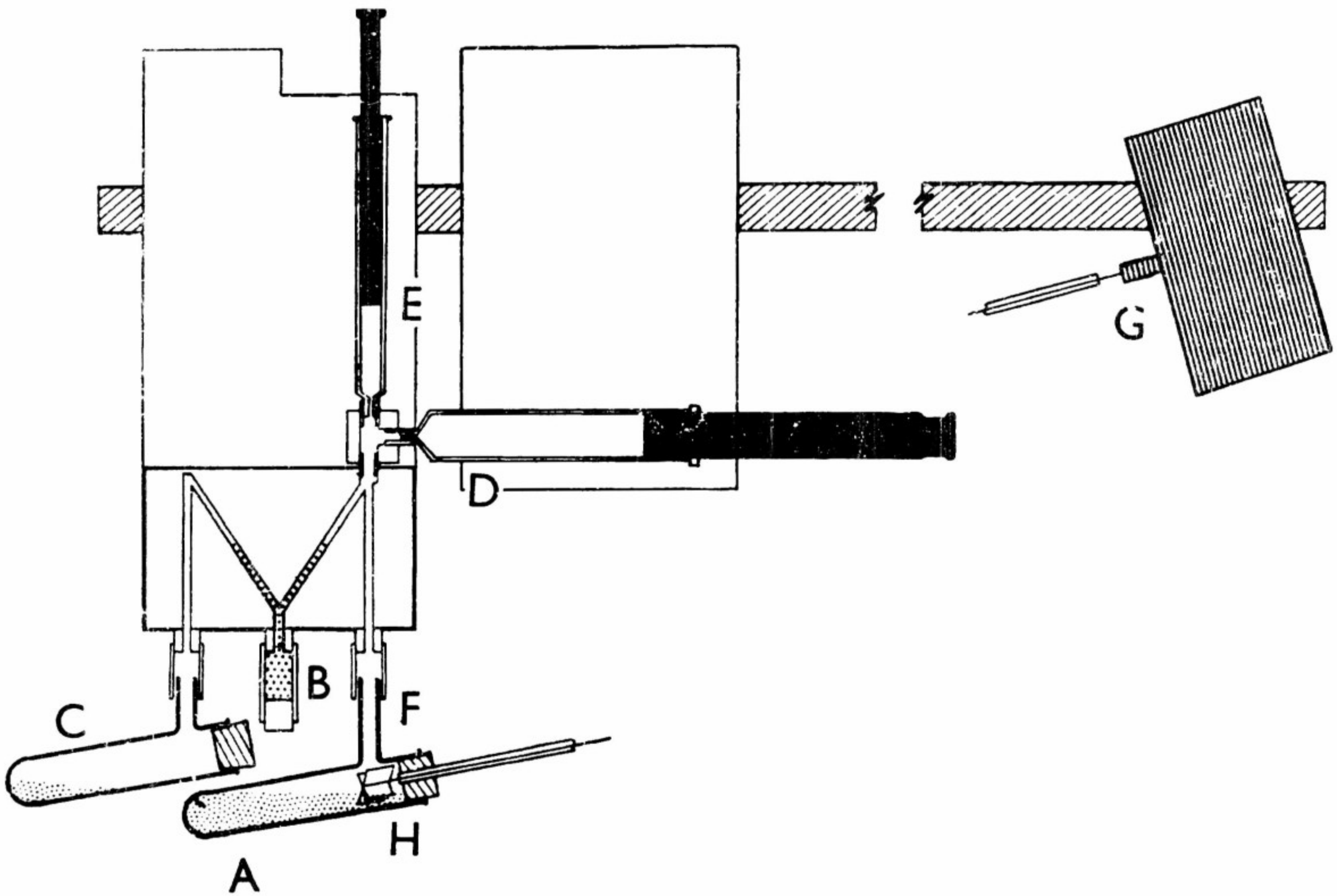

Figure 1. Semi-diagramatic schema of respirometer. A, respirometer vessel; B, reservoir for manometer fluid; C, thermobarometer; D, gas reservoir syringe; E, 1 ml tuberculin syringe, gas reservoir; F, location of carbon dioxide absorbent; G, stirrer motor to power the turbine, H.

As the animal extracts dissolved oxygen from the liquid phase a diffusion gradient is established as a consequence of which additional oxygen diffuses into the aqueous phase from the gas phase. This results in a small but detectable decrease in the pressure of the gas phase. The pressure change is indicated by manometer incorporated in the system. By introducing additional air from a calibrated syringe, which forms part of the closed system, it is possible to restore pressure equilibrium and at the same time, to have an exact, volumetric measurement of the gas consumption by the respiring organism. A second vessel is attached to the manometer block as a thermobarometer. The volume of this is as large as possible to insure maximum sensitivity.

A rheostat-regulated laboratory stirring motor is used to power the turbine. The shaft is stainless steel wire of 0.016" diameter. This is carried in a glass "shaft log" with a bore of 0.020 inches. The latter is packed by blowing full of light, nonoxidizing grease after the shaft is in place.

In use the entire assembly is immersed in a water bath with an observation window. Bath temperature is maintained at 25.0°C. by a combination of heating elements and cooling coils, all under precise control. Temperature sensitivity of the system is very great -- if one simply touches the thermobarometer with the tip of the finger when the system is closed and immersed in its water bath, there results an immediate and extensive alteration of the manometer level. This, of course, is the result of slight heating of the enclosed gas.

After having established the utility of the method by various searching tests, it was applied to a study of the respiration of intact surviving _Teredo_, both _in situ_ and after having been carefully removed from wood. The curve, (Figure 2) is representative of a series of at least twenty similar

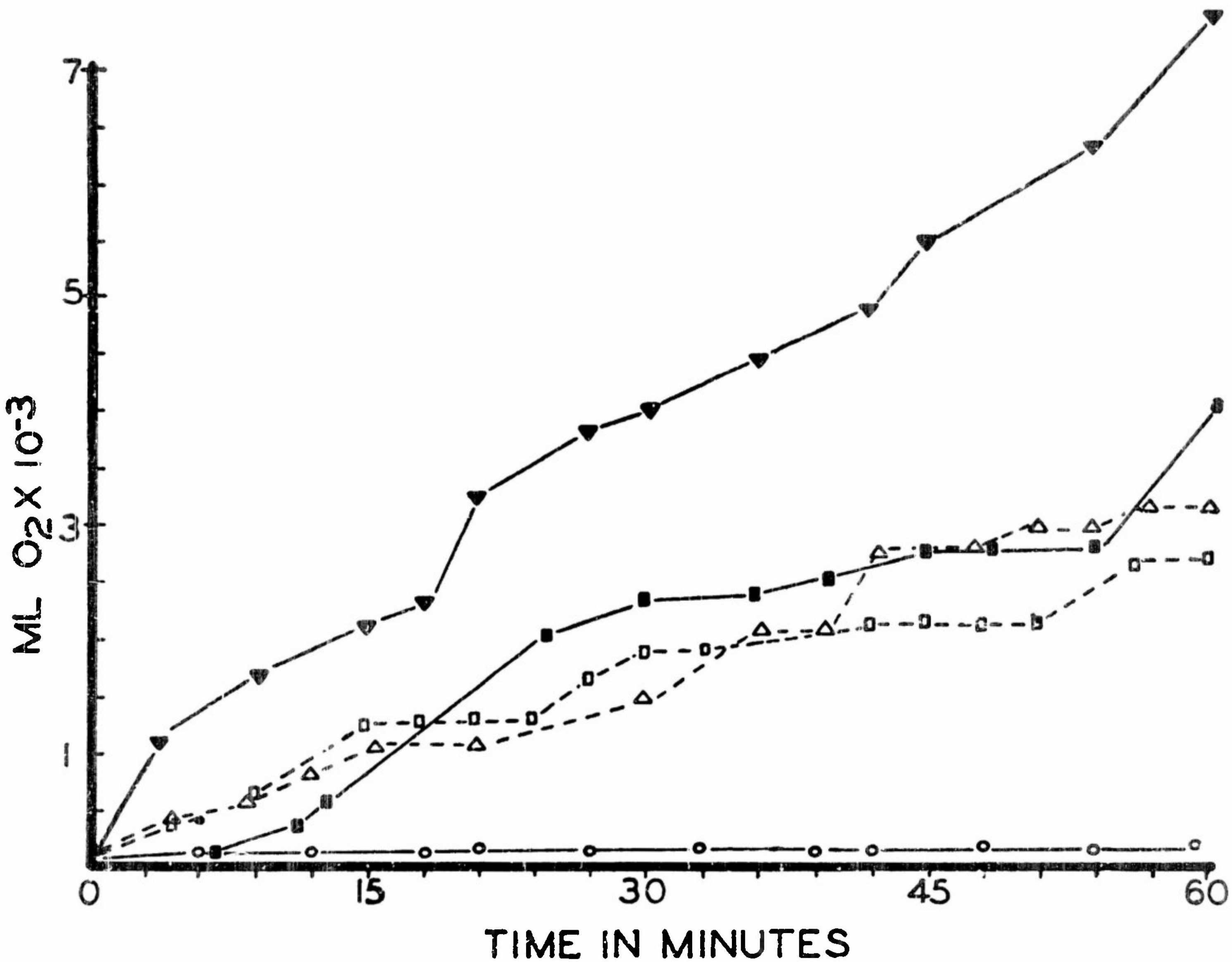

Figure 2. Rate and range of respiration of Teredo, both in situ and after having been removed from wood. Solid lines and conventions delimit range of variation in oxygen consumption of Teredo in wood. Hollow conventions and broken lines delimit range of oxygen consumption for animals out of wood. Lowest line is the sea water blank.

determinations. Here the solid conventions and the unbroken lines represent the rate of oxygen consumption of animals in place, the other curves show the oxygen consumption of animals removed from their burrows.

The oxygen consumption of adult Teredo removed from wood shows considerable variation when expressed as QO_2 (ml O_2/gm dry weight/hr). An average-sized borer, for example, with a dry weight of 75.3 mg. showed a QO_2 of 161.0, while a smaller specimen with a dry weight of only 27.6 mg. showed a QO_2 of 272.5. This inverse relationship between body weight and oxygen consumption was found to be true of all Teredo studied. It should be noted that the figures given above are for animals out of wood, for it is only for such animals that accurate dry weight figures may be obtained. Although their respiration is certainly not typical of that of normal animals. it is reasonable to assume that the QO_2 weight relationship remains, in general, undisturbed.

It will be seen from Figure 2 that there is a slight but unvarying tendency for animals within the wood to respire at a higher rate than those forms which have been removed from their burrows. There is also a tendency for animals in both conditions to respire in a rhythmic, cyclic fashion. It appears that the respiratory pattern of Teredo includes a cycle beginning with hyperoxygenation, following by increasing anoxia which finally triggers the respiratory mechanism to bring about the succeeding period of hyperoxygenation. It is probable that this behavior is associated with the activities of sphincter muscles in the siphons, which alternately restrict and accelerate the flow of the cilia-induced respiratory current of water.

Apparatus for the study of oxygen consumption by individual larvae of Teredo was also designed and used, (Figure 3). It consists of a pear-shaped chamber blowing in one end of capillary tubing. The volume of the chamber

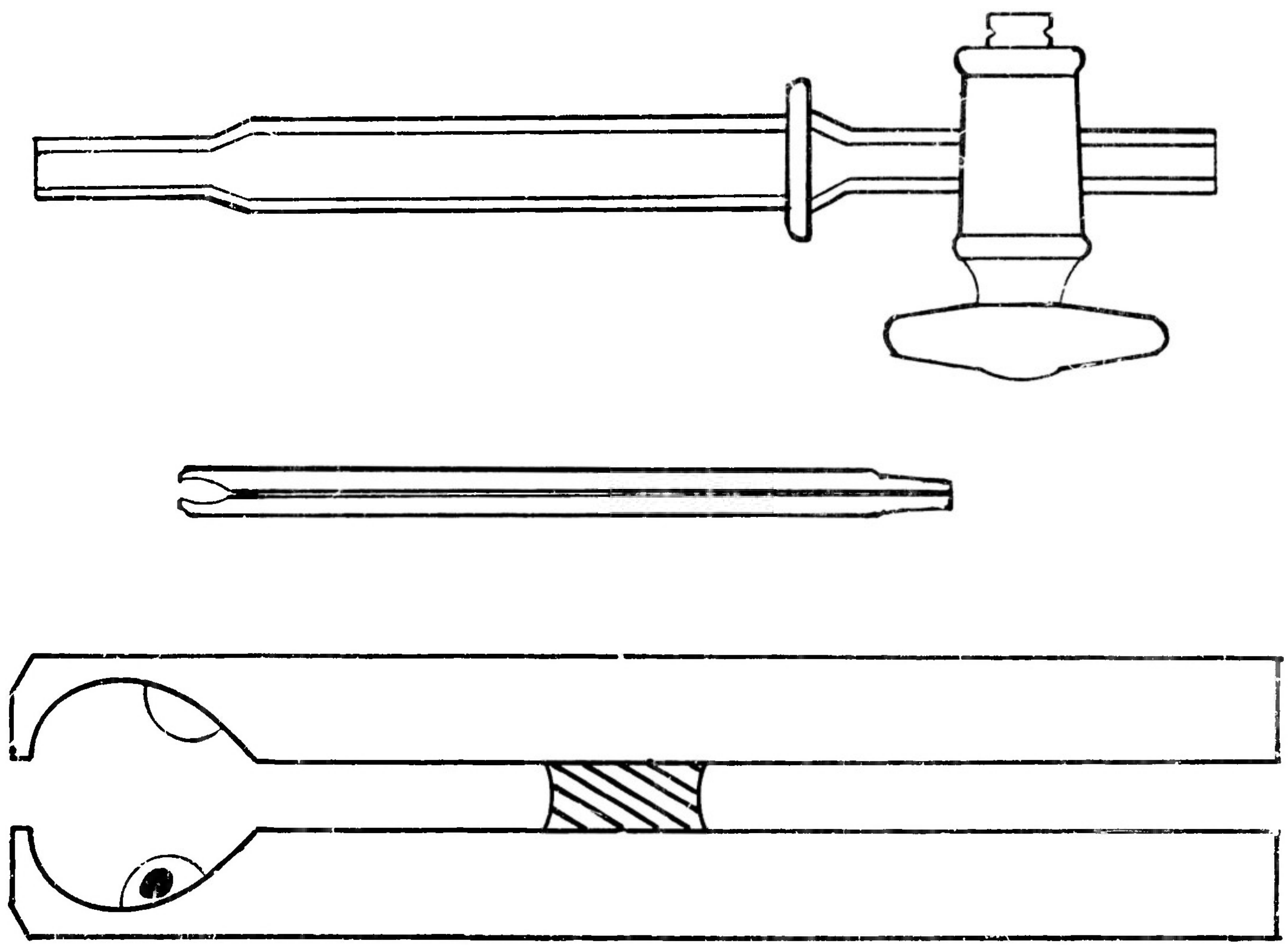

Figure 3. Semi-diagramatic schema of capillary microrespirometer. Enlarged view shows the disposition of the droplets in the respirometer itself.

varies in different respirometers over the range of six to 125 microliters. The volume should be kept as small as possible to increase the stability of the system. The opposite end of the capillary tubing bears an inside syringe-taper ground joint. This seats in the outer matching ground joint of the thermobarometer or compensation chamber. This latter portion of the apparatus should be as large as is consistent with ease of manipulation. We have generally sought to have its volume at least 1,000 times that of the respirometer chamber. This insures maximum sensitivity of the system. The upper end of the compensation chamber is closed by a stopcock. In use the entire assembly is immersed in a constant temperature water bath maintained at 25.0°C.

The chamber is first loaded with a single animal confined in a droplet of medium whose volume varies for different respirometers between one and ten microliters. This volume provides a mass of medium from 100 to 1,000 times the volume of the organism. The isolation of the larva and the determination of the volume of the medium can be effected most easily by making use of specially drawn and calibrated micropipettes. These may be actuated either by a syringe device or by a mouthpiece similar to that of a hemocytometer pipette. Various loading pipettes may be calibrated to deliver precisely known total volumes. The delivered volume, of course, will include the volume of the organisms. Separate pipettes are constructed for each respirometer, and are then used only with that particular apparatus.

The droplet of medium and larva is delivered onto one wall of the respirometer chamber. The chamber wall is previously rendered hydrophobic by the application of a suitable silicone coating. Under these conditions the integrity of the droplet of medium is retained for long periods of time.

It has, for example, frequently been possible to make continuous observations of the oxygen consumption of a single larva during periods as long as twenty-four hours without opening the sealed system.

After the respirometer has been changed with the animal and medium, a droplet containing one to five microliters of alkali, is placed on the contralateral wall. The indicator fluid in the capillary is kerosene which has been distilled at 250°C. after exhaustive oxidation with concentrated sulfuric acid for several days. The open end of the respirometer is sealed with a nonoxidizing wax. For best adhesion and complete sealing it is preferable to employ a wax of comparatively low melting point. With the upper stopcock of the compensation chamber open, the two portions of the apparatus are united, seated, and the joint is sealed with the same wax which was used to close the lower end of the respirometer. The assembly is then placed in the water bath and permitted to come to temperature equilibrium.

With the system sealed and equilibrated the mode of operation is as follows. The larva extracts dissolved oxygen from the sea water medium. This creates a diffusion gradient across the air-water interface, as a consequence of which additional oxygen diffuses into the water from the air phase. The loss of oxygen from the air causes a decrease in pressure in the air phase which is reflected in displacement of the kerosene meniscus. The position of the meniscus is observed with a compound microscope equipped with a long-focus objective. It is clear that the sensitivity of the apparatus is limited by the resolving power of the optical system. In our studies we have found it convenient to detect a displacement of ten microns. This represents a change in volume of 0.002 cubic millimeters. The sensitivity of the system could be increased either by increasing the magnification of the optical system or by decreasing the diameter of the capillary out of which

the respirometer is constructed. For the present study sufficient sensitivity was provided by the dimensions described.

Results secured with this equipment are shown in Figures 4 and 5. Average oxygen consumption values for over one hundred individuals are shown in Figure 4. The lowest curve is the sea-water blank. The middle curve shows the average rate and magnitude of oxygen consumption by normal 24-hour larvae. The upper curve shows the oxygen consumption by normal 24-hour larvae when the sea water medium had been rendered 0.001 M by the addition of appropriate amounts of glucose. Figure 5 shows the change in rate of oxygen consumption with increasing age of the larvae. The increase during the first twenty-four hours is read and significant. After twanty-four hours there is a relatively steady decrease in respiratory rate until death, which occurred in all the animals denied access to wood by 300 hours.

Table III

Oxygen Consumption of Normal *Teredo* Larvae

Age in hours	Number	Average oxygen uptake in mm^3
0	14	61.38
24	14	71.81
48	2	52.80
72	3	35.18
96	3	39.60
120	2	39.60
200	1	33.00
275	1	16.50

The increased oxygen consumption at twenty-four hours may be correlated with behavioral changes in the free-swimming larvae. It has been shown that this stage of development is characterized by a change from a predominantly swimming mode of progression to one in which the chief locomotor activity is crawling with the aid of the muscular foot. The enhanced oxygen uptake at this stage may also be related to the postnatal develop-

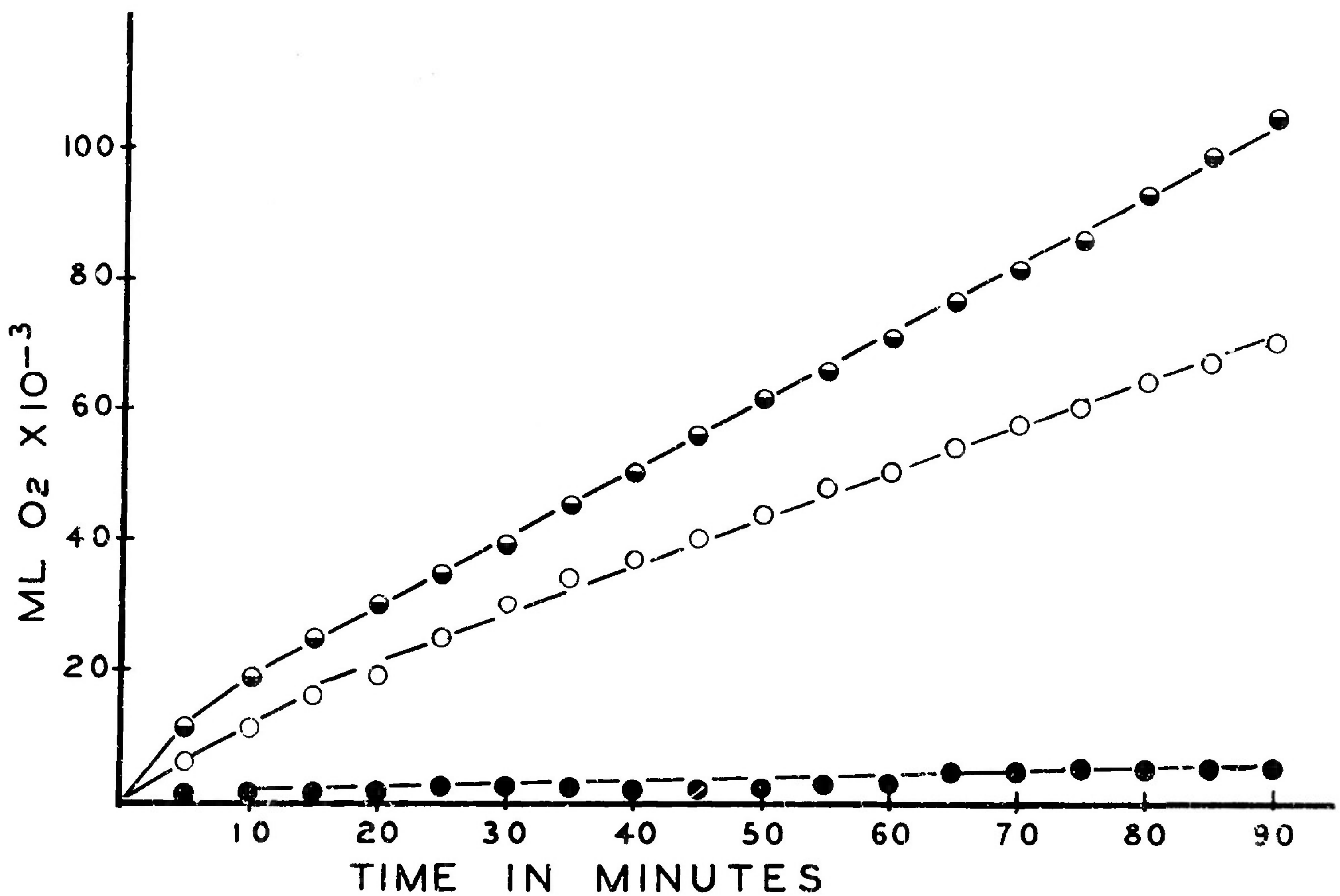

Figure 4. Average oxygen consumption by individual _Teredo_ larvae.
Upper curve is normal 24 hour larvae in M/1000 glucose.
Middle curve is normal 24 hour larvae in normal sea water.
Lower curve is sea water blank.

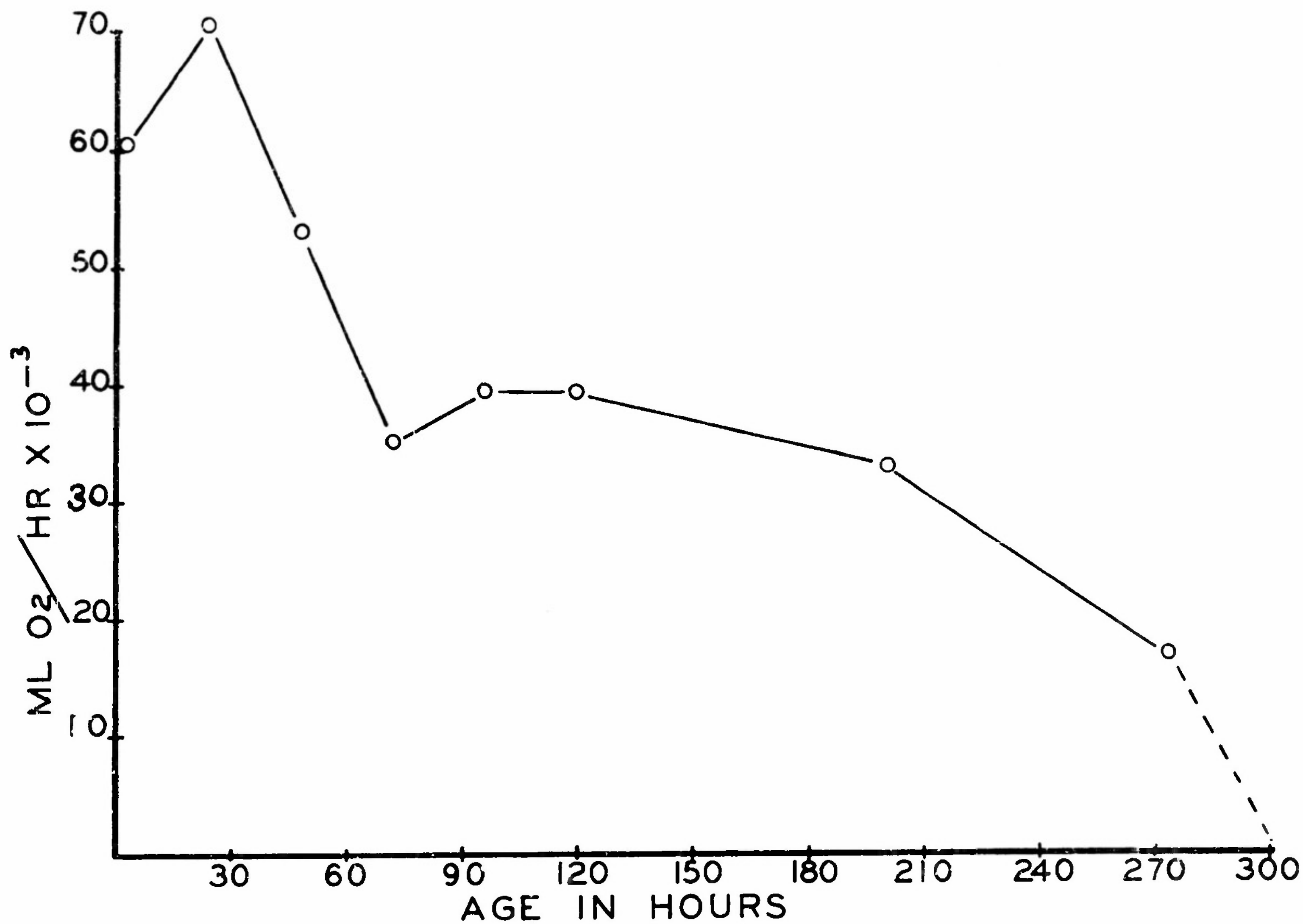

Figure 5. Oxygen consumption vs. age in larvae of _Teredo._ Each plotted point is the average of all determinations made on this age group. Results are shown for 53 separate larvae.

ment of enzymatic mechanisms for complete glycolysis which have not been in existence up to this point in development.

Pumping Rate of Adult Teredo. In earlier work on surviving intact *Teredo in situ* it was repeatedly observed that the sea water in which the animals were maintained was rapidly clarified of all suspended materials. This observation appeared to offer some possibility of determining the rate at which the animals pump water through the mantle cavity. If it be assumed that the water is completely cleared of suspended materials in a single passage through the mantle cavity, then it would be possible to calculate the volume of water pumped by determining the rate at which a known concentration of suspended particles is removed from the medium. To test this hypothesis a suspension of baker's yeast in sea water was made up. Cells were counted and the concentration per unit volume was determined. At hourly intervals samples were withdrawn from the cultures and the concentrations of cells was determined. Control determinations were run simultaneously to determine the number of yeast cells lost by adherence to the glass and to the wood surfaces.

Pumping rate is determined by the use of the following equation:

$$P.R. = \frac{V}{T} \log_e \frac{N_o}{N_t}$$

where:

V = Volume of yeast cell suspension

T = Time

N_o = Original number of yeast cells

N_t = Number of yeast cells at the end of time T

Over fifty determinations were made on eight different *Teredo*. They were selected to span as great a range of size as possible. The size was determined, in each case, by measurement of the X-ray image.

The results indicated that individual shipworms circulate between 4 and 6 ml of water per hour through the mantle.

One further fact of interest appeared from this study. The yeast cells apparently were actually ingested by the borers. Microscopic examination of the fecal pellets of the "fed" borers for thirty-six hours after their yeast meal showed no recognizeable yeast cells to be present. Thus, it is apparently possible for the borers to extract suspended planktonic forms from the respiratory stream and presumably to make use of them for food. No increased oxygen consumption such as would result from specific dynamic action of yeast protein could be detected during the forty-eight hours immediately following such an experiment.

Pumping rate may also be determined by the rate at which the shipworms remove dissolved oxygen from the sea water in which they live. For this purpose use is made of a direct reading volumetric respirometer designed and constructed in this laboratory. It is assumed that the sea water is saturated with oxygen at the temperatures at which the studies are made, and that the animal extracts all the oxygen from the water in each pass over the respiratory surface. The latter assumption is probably not justified by the fact; it is made, however, in order that all observations may be referred to a common datum plane.

Results obtained by these two entirely different methods agree within 7.4% on the average. The total volume of water pumped per shipworm, in our entire series, ranged from 3.6 ml per hour to 13.63 ml per hour. This volume of water exceeds the average volume of the shipworm by approximately a thousand times. The data seem to suggest that the smaller worms pump a greater volume of water per unit of mass than the larger worms.

A third and still more direct approach to the problem of pumping rate in Teredo involved the construction of a vessel in which the incurrent and the excurrent siphons could be separated by a water-tight septum and thus placed into contact with entirely different water masses, which were continuous only through the mantle cavity of a single shipworm. This method confirmed the results obtained by the use of the other two methods.

Hydrodynamic Mechanism. It will be recalled that animals in wood showed a higher rate of respiration than those which had been carefully removed from wood. In order to explain this difference a study of the hydrodynamics of the system was undertaken.

Intramantle pressures were measured in animals *in situ* and out of their burrows. These measurements were effected by the use of #20 to #26 gauge hypodermic needles connected by means of inelastic tygon catheter tubing to a water-filled capillary manometer. *Teredo* in their normal habitat showed a positive intramantle pressure of from 5 to 17 mm of water. The average positive pressure of animals out of wood was less than two millimeters of water. It was noted that the intramantle pressure of normal animals *in situ* tends to show the same cyclic variation as was noticed in the rate of oxygen uptake.

This positive pressure within the mantle is probably of fundamental significance to *Teredo*. It represents the only mechanism for maintenance of the internal turgor which is required to keep the boring end of the flaccid animal in close apposition to the head of the burrow. It also insures that the animal have some control over the circulation of environmental water through its mantle cavity.

In life this positive pressure is insured by the presence of the calcareous lining of the burrow and by the presence of sphincter muscle fibers

in the excurrent siphon. These two factors, combined with the constantly beating cilia of the gill and of the mantle are sufficient to account for the positive pressures which have been repeatedly observed. When *Teredo* is evicted from its burrow one of the factors necessary to the maintenance of this positive pressure - the unyielding wall of the burrow and the calcareous lining thereof - is removed. The first consequence of this is that the volume of the respiratory stream is decreased, bringing about a necessary decrease of oxygen consumption. The other consequence is that the mantle develops herniations. The mantle itself is virtually devoid of muscle elements or of any other specific supporting elements. Thus, unsupported by shell or the wooden wall of the burrow, it is incapable of counteracting the pressure which exists within it, The result is the development of from one to several herniations in areas of structural weakness of the mantle.

Cellulase Enzyme System. Since *Teredo* inhabits wood, and since the architecture of its digestive system dictates that wood rasped away by the shells must pass through the gut before it can be eliminated from the burrow, it has long been assumed that Teredo possesses a cellulase enzyme system which would permit the animal to make some dietary use of the wood it is forced to ingest. Little experimental evidence seems to have accumulated since the pioneering work of Boynton and Miller in 1927.

Our studies employed homogenized shipworm tissues and regenerated cellulose suspended in Sorenson's phosphate buffer made isotonic with sea water. Isotonicity was achieved by the addition of sodium chloride. This artificial reaction medium provided an opportunity to study the pH optimum of the reaction. The following range was studied: pH 5.0; 5.6; 6.0; 6.7;6.9, and 7.6

The regenerated cellulose used in these experiments was prepared after the method of Parkin. Cellulose fiber sheets are dissolved in a mixture of

zinc chloride and concentrated hydrochloric acid (1:2 by weight). The resulting syrupy liquid is filtered through a column of glass wool. The cellulose is then reprecipitated by the addition of distilled water. The precipitate was washed free of acid, resuspended and then dialyzed overnight to remove salts.

Tissues to be studied were removed from living Teredo, stored briefly in aerated, chilled sea water at 4°C, then homogenized in the buffer solution which was to be employed for the test.

Tissues used included the entire tubular gut and its contents, the anterior half of the gut and content, the gut diverticula, and the anterior end of the animal without gut or gut diverticula. These portions were carefully dissected, then stored in chilled sea water until sufficient material had been accumulated to charge the homogenizer. The tissues were added to the homogenizer in units. The same number of similar units were also taken into another container, dried, and then analyzed for polysaccharide and for reducing sugar.

After incubation for known times at fixed temperatures, the amount of reducing sugar was determined. The method used was that of Park and Johnson which was shown to be sensitive over a range of 1 to 9 gamma of glucose. Glycogen was also determined in each of the incubation mixtures.

Controls employed included the following: 1) tissue alone, 2) tissue plus medium alone, 3) boiled tissue plus medium, 4) Cellulose plus sea water alone. Adequate bacteriological controls were run simultaneously — no significant bacgerial population was ever noted. Colonies that did develop did not exhibit cellulose-digesting ability. Controls were all run in parallel with the experimental determinations, were removed from the incubator at the same time, and were subjected to the same manipulative methods as were the experimental vessels.

Results showed that the entire anterior portion of the gut contains cellulase activity. There is apparently no concentration of activity in any of the gut diverticula.

In common with cellulase enzyme from other sources, Teredo cellulase exhibits a broad pH optimum between pH 5.6 and pH 6.5 . Some evidence exists to indicate that there may be two systems with slightly different pH optima contributing to the total cellulose-digesting ability of Teredo.

Carbohydrate Metabolism. Previous studies on glycogen content in the shipworm, and on its enzymatic armament, and observations of the significant resistance which the borers show to anoxia have led to an investigation of the carbohydrate metabolism of the animal -- particularly that associated with anaerobiosis. The measurement of energy metabolism under these conditions involves quantitative analysis of certain of the intermediates of glycolysis as well as studies of oxygen uptake during active glycolysis.

A qualitative analysis of some of the intermediates was accomplished by the method of paper chromatography of homogenates of entire shipworms. The organisms were extracted from the infected wood and were chilled immediately. The soft tissues were then homogenized and centrifuged. The supernatant fluid was then chromatographed. In this way the following substances were detected: adenylic acid, adenosine polyphosphates (ATP & ADP), hexose phosphates and arginine phosphate. No creatine phosphate could be detected by the methods employed in these studies. Oxygen consumption studies have confirmed the ability of the borers to tolerate conditions of low oxygen tension for periods of at least one week.

CRESOTE STUDIES

Biological Assay Procedures.

Capillary Microrespirometer. Having established a method for determining oxygen consumption by a single Teredo larva, and having established

the normal range of variation in oxygen uptake over the entire free-living life of the animal, it was then determined to study the effect of standard whole creosote upon the respiration of individual larvae.

Creosote was homogenized with sea water in a hand homogenizer. The emulsion was at first allowed to stand for 24 hours and the aqueous phase, presumably saturated at ambient temperature and pressure, was diluted with fresh sea water. In later work the initial emulsion was employed without the twenty-four hour waiting period. Dilution of 1×10^{-6} produced a solution which killed all larvae within 60 minutes. Dilution of 1×10^{-7} permitted survivals of several hours. This period was long enough to make possible a respirometer run before the death of the larva. This dilution contained 5×10^{-8} gms creosote/ml sea water. When one 12-hour old Teredo larva is placed in 10 microliters of this medium the oxygen uptake is reduced on the average by 68.8 percent. Further dilutions were employed to determine the minimal level at which a response could be detected.

Table IV

Effects of Creosote on Respiration of *Teredo* Larvae

Concentration of Creosote	Average Oxygen Uptake/hr./larva
5×10^{-7} gm/ml	17.13 mm^3
5×10^{-8}	21.15
5×10^{-9}	28.70
5×10^{-10}	34.75
5×10^{-11}	41.70
5×10^{-12}	45.40
0.0 (normal sea water)	47.8

Table IV includes average values derived from a study of 10 animals at each concentration level. Normal figures are average of thirty normal animals in sea water, so this table presents average results of a study of 90 individual *Teredo* larvae. These results provide a pharmacologic standard of reference against which to compare the toxicity, not only of creosote fractions, but also of any other toxic material which may be prepared in the future. Our figures show that a concentration of whole creosote between 5×10^{-11} and 5×10^{-12} gm/ml sea water is the minimal level at which a response can be detected under the conditions of our experiments. Concentrations of the order of 5×10^{-7} gm/ml sea water are uniformly lethal in eight hours.

During the completion of these studies it became increasingly clear that the capillary microrespirometer has certain shortcomings for routine, large-scale screening operations. Thus, it is possible to study but one animal at a time; the time required for thermal equilibration often exceeds two hours. Finally the instrument is too sensitive for routine use. For these reasons some attention was early devoted to the possibility of employing the standard Warburg respirometer. As has been mentioned before in this final report, this apparatus is not suitable for oxygen uptake studies in *Teredo* because of the agitation which is required. However, *Limnoria*, in some initial and unreported experiments, showed complete tolerance of the oscillations of the Warburg apparatus. Further investigation of this form showed that the combined oxygen uptake of 25 adult *Limnoria* during a period of one hour, is sufficiently large to be measured handily with this technique. It remained only to demonstrate that whole creosote produced a measureable effect upon this total oxygen consumption. A series of dilutions of whole creosote, prepared by the methods standard for these studies, was prepared. Table V shows the results of this study.

It will be observed that the effect, which is real, significant and reproducible, is a definite augmentation of the oxygen uptake of the experimental animals. It is thought that this added oxygen consumption provides a measure of the metabolic work being done in detoxification. It has frequently been reported that Limnoria prosper in wood which has been treated with creosote so they must possess metabolic machinery for rendering it innocuous. It is apparent from the results shown in the table that the magnitude of the response is, in general, a function of the concentration of whole creosote which is present in the medium.

Having thus demonstrated the reality of the physiological reaction to creosote in Limnoria, and the relative utility of Limnoria as a test animal, some attention was then devoted to a study of the toxicity of creosote fractions supplied us by the Naval Research Laboratory.

Since whole creosote produced a maximal reaction when it was diluted 1:10,000 with sea water, it was determined to employ this same dilution in the assay of creosote fractions. This procedure admittedly does not compare the toxicity of whole creosote with the toxicity of its component fractions when these are applied alone in the concentration at which they were present in the whole sample. Information on the quantitative compositions of the original creosote sample was not available to us at the time when the tests were initiated.

It will be observed in Table V that "creosote with tar acids and tar bases removed", "aqueous alkali extract of whole creosote" and m/1,000 glucose in sea water are all about equally effective in increasing the oxygen consumption of Limnoria. On the other hand the two fractions which were produced by solvent extraction produced approximately the same respiratory re-

Table V

Effect of Various Agents on Oxygen Consumption

by Limnoria**

Material tested	Dilution in sea water	Number of tests	Oxygen uptake*	% Increase over normal
Control determinations		47	11.935±2.83	0
M/1,000 Glucose in sea water		10	14.267	19.
Whole Creosote	$1:10^6$	25	16.033	34.2
Whole Creosote	$1:10^4$	19	21.249±3.90	77.5
"Solvent Extraction fraction, Residue after removal of solids only"	$1:10^4$	15	16.955	41.9
"Solvent Extraction fraction #152.5 (B158)	$1:10^4$	9	20.810	74.2
"Aqueous Alkali Extract of Whole Creosote	$1:10^4$	15	13.640	14.3
"Creosote with Tar Acids and Tar Bases Removed"	$1:10^4$	18	14.960	25.2
Creosote with Tar Bases Removed"	$1:10^4$	14	23.920±2.56	100.5

* Oxygen consumption is expressed as mm^3/hr/group of 25 animals
** All determinations were made using twenty-five Limnoria per Warburg flask

sponse as whole creosote at comparable dilution. "Creosote with tar bases removed" was easily the most effective fraction studied, resulting in an increase of over 100% in oxygen uptake over the rate shown by untreated normal control animals.

<u>Accelerated Leaching Tests</u>. <u>Summary of Creosote-Treated and Miscellaneous Wood Panel Exposure Tests</u>. On the assumption that creosote-treated wood liberates a soluble toxic substance in seawater an extensive series of panel exposure tests have been conducted since 1949.

Marine borer larvae were used in the first tests of creosoted wood. Active larvae were allowed to crawl about on both creosote-treated slips and on control slips of untreated wood. While the larvae crawled about constantly on the control slips they appeared much more sluggish on the creosote panels. After three days exposure to the creosote panels none of the larvae recovered when transferred to fresh seawater. For periods of exposure up to three days the larvae recovered when transferred to fresh seawater, the period of recovery varying with the length of exposure. It was further found that the larvae would only bore into the untreated slips. However, in the presence of creosote-treated slips, the untreated slips were not attacked.

It was decided to study the rate at which the toxic properties of the creosote-treated wood are lost when subjected to accelerated leaching conditions. Accelerated leaching techniques could prove valuable in simulating long service exposure of dock timbers.

Coupled with the accelerated leaching tests a general program of investigation into the active constituents of creosote was initiated in 1951 in cooperation with the Naval Research Laboratory. The accelerated leaching of each of the creosote constituents would give an index of the comparative value of each constituentand of the whole creosote. It would also give an idea of the resistance of a treated panel when first exposed and its probable length of service life.

The first accelerated leaching tests were conducted on slips 6 x 2 x 1/8 in. and subjected to various baths as well as being placed directly in the ocean. The baths included boiling sea water, cold alkaline potassium dichromate, hydrochlric acid, aerated seawater and sodium hydroxide. After testing the action of the larvae on the slips subjected to the various treatments it was found that aerated sea water and boiling sea water decrease the

inhibitory properties of creosoted wood but that the acid, alkali and oxidizing baths are ineffective. It was further found when the panels were exposed in the ocean to borer attack that the only effective treatment was boiling sea water. For convenience and constancy a bath at 90°C was used instead of boiling water. This was ultimately changed to flowing fresh water and the temperature dropped to 80°C.

It was determined that after two weeks leaching with fresh water at 80°C wood with a 20 pound creosote retention lost its resistance to marine borer attack. As this normally does not happen until ten to twenty years of normal service life has elapsed a definite relationship may be set up between the time of leaching and actual service life. The test slips were reduced in size to 5 x 1½ x 1/8 inches.

In December of 1952 a new apparatus was constructed for the purpose of leaching creosote slips. It consisted of a large 25 gallon stainless steel rectangular tank with a capacity of 200 slips. It is outfitted with a close-fitting cover and equipped with a heavy duty heater, thermostats, two stirrers and is connected to a fresh-water source which permits a continual flow of water through the tank. The thermoregulators are located near each end of the tank so that an accurate control of the temperature is possible.

It was found in previous tests that a leaching period of 16 days was optimum. At the 16 day period resistance to borer attack proved to be a positive function of the creosote concentration in the panel.

Leaching periods of less than 16 days did not show consistent correlations. Leaching periods of more than 16 days proved to be too long since panels with a low concentration of creosote would not last for the six months test exposure period. Six months (field exposure) was chosen to allow for the seasonal variation in attack rate.

However, leaching periods of 0, 2, 4, 8, 16, 32, 48, 60, and 72 days were used to further substantiate the choice of the 16 day leaching period as the optimum. Test panels were impregnated with creosote and the various fractions of creosote in the range of approximately 2 pounds per cubic foot to 33 pounds per cubic foot. The fractions tested included the following: Claisen-alkali extracted creosote, (the residue of which, in the amount of 94.4% of the original creosote, was used to impregnate the panels); Distillation fraction No. 2 (boiling range 200° to 235° at 760 mm) which amounted to 19.5% of the whole creosote but which was fortified so that it contained double the concentration of this fraction, i.e. 39% and the panels impregnated with this; distillation fraction no. 3(b.r. 235 at 760 mm to 130° at 15mm) which amounted to 6.4%, or double, 12.8%; distillation fraction 4 (8.9% enough of which was added to whole creosote to double the % of the fraction or 17.8%; fraction 5 (10.1% doubled to 20.2%); fraction 6 (16.5% doubled to 33%) and the residue from the distillation. Other fractions tested were: chlorinated creosote; creosote freed of tar bases (the tar bases amount to 3.5% of the whole creosote); creosote freed of tar acids and bases (this amounts to 9% of the whole creosote); and of course whole creosote panels were exposed simultaneously with each of the above fractions.

In exposures of less than 4 months duration attack-rate varied independently of that prior to four months exposure the extent of attack varied degree of leaching. Attack after four months exposure showed a rapid but erratic increase with amount of leaching up to 16 days. After 16 days leaching, attack rate remains fairly constant, with only a slight positive correlation with leaching period.

The panels were rated from 0 to 5 or from no attack to very heavy attack or complete riddling of the panel. Not only shipworms but also Limnoria was

rated independently of each other. Since Limnoria attack occurs more quickly and proceeds more rapidly than borer attack it was decided to plot the damage as $L + 2T$ or by doubling the borer rating to offset the abnormal Limnoria damage. Thus, damage ($L + 2T$) was plotted as a function of concentration (lbs. retention creosote/ft^3.)

Results of exposures of the above fractions compared to whole creosote showed a considerable range of variation in numerical data. Panels impregnated in the 2 to 5 pound range and leaching periods over 16 days would not hold up over the 6 month exposure period. The initial high resistance of unleached creosote panels did not diminish uniformly as was hoped. These results led to the following changes in the basic protocol.

The entire series of fractions are to be re-exposed. The various fractions will be impregnated into the panels in the restricted range of 10 to 15 lbs/ft^3 while whole creosote will be impregnated in the wider range of 5 to 20 lbs. One set of each fractions will be leached for the optimum period of 16 days and a second set will be exposed unleached. For every two fractions (four sets of 20 panels each) two sets of whole creosote will be exposed simultaneously, one unleached and the other leached for 16 days. In addition control panels, untreated wood of the same type as the treated panels, will be leached for 16 days and together with unleached control panels will be exposed along with the treated panels.

Heretofore the intense fouling of the panels suspended in Biscayne Bay has made the job of cleaning the panels for evaluation difficult and probably has lessened the amount of overall attack or else prolonged the period of evaluation. So, it was decided to scrape the panels free of fouling every two weeks so that maximum ack could take place in the shortest time. Panels will continue to be examined and rated every two months.

At the end of 1953 the following fractions were under test in the bay: claisen-alkali extract; base-free; acid and base free; tar-free; solids-free; tar and solids-free; distillation fraction 2 and fraction 3. These panels will be exposed until they reach a rating of 3 or 4 at which time they will be removed from the water and their total time of immersion noted.

At the same time long term exposure tests are being run using 2 x 4 x 8 inch blocks impregnated with whole creosote at different levels.

Miscellaneous Panel Exposure Tests

Panels treated with hydroxymercurilignin by the Institute of Paper Chemistry did not hold up beyond twelve months without heavy damage taking place. Similar results occurred with cuprinol treated panels (3% copper retention).

Creosote pilings which failed at Guantanamo Bay were tested. It was found that only the outer inch of the piling showed toxicity. The inner part of the timber was completely bored. It was concluded that the effective treatment of these pilings does not extend beyond the outer one inch.

Unleached creosoted blocks with retentions of 26 to 31 pounds have been in the water for over 2½ years with no damage either from marine borers or Limnoria.

A Greenheart timber exposed for over 3½ years shows only very light Limnoria attack and only moderate borer attack.

Surinam timbers (silica-containing) panels have been exposed for 3 years. Some have been destroyed but others have shown very little attack during the total period.

Toxic diffusion tests were accomplished with the aid of so-called holiday panels i.e. a plain untreated panel bolted between two treated panels. Results have shown that anti-fouling copper paint will protect these untreated holidays of as much as ½" thickness and that creosote will protect only 1/8" holidays.

Experiments were conducted to determine the effect of prior immersion in sterile fresh water, sterile sea water and filtered sea water on the attack rate of untreated panels. Panels were immersed for varying periods of 1 to 32 days in each of the above liquids and then exposed in the bay. The results after three months exposure showed no difference among any of the panels.

Erdalith-treated panels exposed for two years showed absolutely no damage while the attendant control panels were completely destroyed.

Composite panels, i.e. an untreated panel bolted to a creosote-treated panel showed that the Limnoria and Teredo attacked and destroyed the untreated panel but did not attack or cross over into the creosote-treated panel.

Artificial cellulose panels were exposed. They showed extensive Limnoria attack with very little borer damage.

Spruce veneer panels exhibited no borer attack after one year. Resin-impregnated and acetylated veneer likewise showed no borer after one year exposure. A zinc chromate - treated panel showed only moderate borer attack after 21 months.

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